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# **Involvement of mitochondrial ferredoxin and para-aminobenzoic acid in yeast coenzyme Q biosynthesis**

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**Running title:** pABA is a precursor of yeast coenzyme Q

## Summary

Yeast ubiquinone or coenzyme Q<sub>6</sub> (Q<sub>6</sub>) is a redox active lipid with a crucial role in the mitochondrial electron transport chain. At least nine proteins (Coq1p-9p) participate in Q<sub>6</sub> biosynthesis from 4-hydroxybenzoate (4-HB). We now show that the mitochondrial ferredoxin Yah1p and the ferredoxin reductase Arh1p are required for Q<sub>6</sub> biosynthesis, probably for the first hydroxylation of the pathway. Conditional Gal-*YAH1* and Gal-*ARH1* mutants accumulate 3-hexaprenyl-4-hydroxyphenol and 3-hexaprenyl-4-aminophenol. Para-aminobenzoic acid (pABA) is shown to be the precursor of 3-hexaprenyl-4-aminophenol and to compete with 4-HB for the prenylation reaction catalyzed by Coq2p. Yeast cells convert U-(<sup>13</sup>C)-pABA into <sup>13</sup>C ring-labeled Q<sub>6</sub>, a result which identifies pABA as a new precursor of Q<sub>6</sub> and implies an additional NH<sub>2</sub>-to-OH conversion in Q<sub>6</sub> biosynthesis. Our study identifies pABA, Yah1p and Arh1p as three new actors in Q<sub>6</sub> biosynthesis.

## Introduction

Coenzyme Q (ubiquinone or Q) is a lipophilic organic molecule composed of a substituted benzoquinone and a polyprenyl chain containing 6 units in *Saccharomyces cerevisiae* (Q<sub>6</sub>) and 10 in humans (Q<sub>10</sub>). Q has a well known role as an electron carrier in the mitochondrial respiratory chain and also functions as a membrane soluble antioxidant (Bentinger et al., 2007). Moreover, Q has been implicated in modulating the permeability of transition pores (Fontaine and Bernardi, 1999) and is an obligatory cofactor for the proton transport function of uncoupling proteins (Echtay et al., 2001).

4-hydroxybenzoate (4-HB) is the long-known aromatic precursor of the benzoquinone ring of Q (Olson et al., 1963; Rudney and Parson, 1963). Cells synthesize Q from 4-HB and from dimethylallyl diphosphate and isoprenyl diphosphate, the building blocks for the polyprenyl chain. After covalent linkage of the polyprenyl tail to 4-HB to form 3-polyprenyl-4-hydroxybenzoate, a total of 1 decarboxylation, 3 hydroxylation and 3 methylation reactions at the aromatic ring are necessary to yield Q (Figure 1). Current knowledge of the genes involved in Q biosynthesis in eukaryotes is mostly derived from the work carried out on yeast by C. Clarke (Tran and Clarke, 2007). So far, 9 genes (*COQ1-9*) have been characterized as essential for Q biosynthesis in yeast but only 6 gene products have been assigned a catalytic role (Figure 1)(Kawamukai, 2009). All *S. cerevisiae* *COQ1-9* genes have human relatives. Primary Q<sub>10</sub> deficiency, a rare recessive disorder, has now been linked to mutations in five genes: *PDSS1* (Mollet et al., 2007) and *PDSS2* (Lopez et al., 2006; Mollet et al., 2007), which are the relatives of *COQ1*; *COQ2* (Mollet et al., 2007); *COQ9* (Duncan et al., 2009) and *ADCK3/CABC1* the relative of *COQ8* (Lagier-Tourenne et al., 2008; Mollet et al., 2008).

Genetic and biochemical data have shown in yeast that a multi-protein Q biosynthetic complex forms and associates with the mitochondrial inner membrane on the matrix side (Tran and Clarke, 2007). The proteins Coq3, Coq4, Coq6, Coq7 and Coq9 are part of the Q

biosynthetic complex (Tran and Clarke, 2007) and steady state levels of these proteins are strongly decreased in any *coq* null mutants that lack one of the *COQ* genes (Tran and Clarke, 2007). This explains why yeast *coq* mutants rarely accumulate biosynthetic Q intermediates that may be diagnostic of the altered reaction in a particular mutant. Indeed, yeast null *coq3* to *coq9* mutants accumulate the same early intermediate 3-hexaprenyl-4-hydroxybenzoate (HHB) (Figure 1). Only selective mutations altering the activity of a Coq protein without drastically affecting assembly of the Q biosynthetic complex may lead to accumulation of intermediates different from HHB. Only 2 such intermediates have been unambiguously identified and linked to a mutation in a *COQ* gene: 5-demethoxyubiquinone (DMQ<sub>6</sub>) accumulates in two *coq7* mutants, L237Stop and E233K (Padilla et al., 2004), and 3-hexaprenyl-4,5-dihydroxybenzoate accumulates in an uncharacterized *coq3* mutant strain (Clarke et al., 1991). The rare occurrence of intermediates diagnostic of the defective step in Q biosynthesis largely explains why the order of reactions modifying the aromatic ring of 4-polyprenyl-3 hydroxybenzoate is still speculative (Tran and Clarke, 2007).

Coq6p and Coq7p are two mono-oxygenases that function of Q biosynthesis. Coq7p hydroxylates DMQ<sub>6</sub> (Padilla et al., 2004; Tran et al., 2006) and Coq6p may catalyse one or both of the two remaining hydroxylation steps (Gin et al., 2003) (Figure 1). Mono-oxygenases are widely distributed proteins that catalyze incorporation of one oxygen atom into substrates. Dioxygen is the source of the oxygen atom and mono-oxygenases usually employ a transition metal (iron, copper) or an organic cofactor (flavin, pterin) to mediate dioxygen activation at their active site. This activation requires two electrons which are provided by a reductant. NAD(P)H can directly reduce the flavin cofactor of flavoprotein mono-oxygenases whereas reduction of the heme iron in cytochrome P450 type oxygenases is accomplished by an associated reductase (Ballou et al., 2005; Munro et al., 2007). The mammalian electron transport machinery of mitochondrial cytochrome P450 consists of two proteins, an NADPH-

ferredoxin reductase called adrenodoxin reductase (AdxR) and a [2Fe-2S] ferredoxin called adrenodoxin (Adx). To date, proteins resembling mitochondrial cytochrome P450 mono-oxygenase have not been found in yeast, yet yeast expresses a mitochondrial ferredoxin (Yah1p) and a mitochondrial ferredoxin reductase (Arh1p) which are homologues of Adx and AdxR.

The Yah1p/Arh1p system is located in the mitochondrial matrix and is required for viability (Barros and Nobrega, 1999; Manzella et al., 1998). Yah1p and Arh1p play an essential role in the biogenesis of Fe-S clusters (Lange et al., 2000; Li et al., 2001) and of heme A (Barros et al., 2002), an indispensable cofactor of cytochrome *c* oxidase. Cox15p which catalyzes the conversion of heme O to heme A is the likely recipient of electrons transferred by Yah1p (Barros et al., 2002) whereas the recipient is still unknown for the biogenesis of Fe-S clusters. Conserved motifs in the primary sequence of Coq6p and Coq7p predict that these two mono-oxygenases employ a flavin and a di-iron cofactor respectively (Gin et al., 2003; Stenmark et al., 2001). The oxidized flavin of Coq6p is a two-electron acceptor whereas the oxidized di-iron ( $\text{Fe}^{\text{III}}\text{-Fe}^{\text{III}}$ ) center of Coq7p will also accept two electrons but one at a time. Therefore the putative direct two-electron reduction of the flavin of Coq6p by hydride transfer from NAD(P)H is not applicable to the di-iron center of Coq7p. The *in vivo* source of electrons used by Coq7p is still unknown and we hypothesized that the Yah1p/Arh1p system may fulfill this role.

In this work, we show that the Yah1p/Arh1p system is essential for yeast  $\text{Q}_6$  synthesis. Cells depleted for Yah1p or Arh1p accumulate specific intermediates of  $\text{Q}_6$  upstream of  $\text{DMQ}_6$ , the substrate of Coq7p. The accumulation of these early intermediates pointed to the involvement of Yah1p/Arh1p in the first hydroxylation reaction of  $\text{Q}_6$  biosynthesis. Surprisingly, one of the intermediates accumulated in Yah1p-depleted cells contains an amine group. This observation led us to show that para-aminobenzoic acid (pABA) is the precursor

of this intermediate and that pABA enters the Q biosynthetic pathway at the prenylation reaction catalyzed by Coq2p. Moreover, labeling experiments proved that pABA is an *in vivo* precursor of the benzoquinone ring of Q<sub>6</sub>. This new, unexpected role of pABA has been overlooked since the discovery of 4-HB as the precursor of the benzoquinone ring of Q more than 40 years ago.

## Results

### **The mitochondrial ferredoxin Yah1p and its associated reductase Arh1p are essential for Q<sub>6</sub> biosynthesis**

In order to test the possible role of the mitochondrial ferredoxin Yah1p in Q<sub>6</sub> biosynthesis, we used the strain Gal-YAH1 in which the native YAH1 promoter was replaced by the GAL10 promoter (Lange et al., 2000). Therefore, the expression of the essential YAH1 gene in the Gal-YAH1 strain is under the control of a galactose-inducible promoter (Lange et al., 2000). When YAH1 expression was repressed by cultivating the Gal-YAH1 strain in a glucose-containing medium, we noted a strong depletion of the Q<sub>6</sub> content of the cells together with that of DMQ<sub>6</sub> (Figure 2A). Q<sub>6</sub> and DMQ<sub>6</sub> were detected electrochemically after separation of cell lipid extracts by HPLC. Surprisingly, we detected an additional electroactive compound eluting at around 610 seconds which was absent from the galactose-grown cells (Figure 2A). The striking decrease of Q<sub>6</sub> does not result from dying cells because the cells were viable at the end of the glucose culture as assessed by determining the colony forming units on galactose medium. Introduction of a plasmid carrying the YAH1 gene in the Gal-YAH1 strain restored wild-type Q<sub>6</sub> levels in glucose-grown cells and abolished the accumulation of the electroactive compound (Figure 2B). To verify that the [2Fe-2S] cluster of Yah1p was required for Q<sub>6</sub> generation, we constructed mutant alleles of Yah1p in which conserved Fe-S

cysteine ligands (C103 and C106) are mutated to alanine (Figure S1) (Xia et al., 1996). The electrochromatograms of extracts from glucose-grown Gal-*YAH1* strains containing an empty vector and from C103A or C103-106S mutants of Yah1p were similar, with an accumulation of the newly detected metabolite, showing that these cysteines are essential for Yah1p function (Figure 2B and data not shown). The Fe-S center of Yah1p is reduced by electrons provided by the ferredoxin reductase Arh1p which is also an essential protein (Manzella et al., 1998). In order to corroborate the observation that Yah1p is required for Q<sub>6</sub> biosynthesis, we generated a Gal-*ARH1* strain in which the expression of *ARH1* is repressed by growth in glucose medium. After growth of the Gal-*ARH1* strain in glucose medium, Arh1p was undetectable in purified mitochondria but Yah1p steady state level was only mildly decreased (Figure 2C). These mitochondria showed little Q<sub>6</sub> and accumulated the electroactive compound found in Yah1p-depleted mitochondria (Figure 2D). Altogether, these results show that the absence of a functional ferredoxin / ferredoxin reductase system strongly depletes cellular and mitochondrial levels of Q<sub>6</sub> and promotes the accumulation of a novel compound.

### **A strain deficient in Yah1p accumulates 3-hexaprenyl-4-aminophenol, a product derived from the Q<sub>6</sub> biosynthetic pathway**

We attempted to identify the origin and nature of the electroactive compound accumulating in Yah1p-deficient cells and found that it is different from DMQ<sub>6</sub>, the only intermediate of Q<sub>6</sub> biosynthesis observed by electrochemical detection so far. Deletion of *COQ5* or *COQ7*, two genes essential for Q<sub>6</sub> biosynthesis, abolishes formation of Q<sub>6</sub> and results in the accumulation of HHB because many Coq proteins of the Q biosynthetic complex are unstable in these mutants (Figure 1)(Tran and Clarke, 2007). Deletion of *COQ5* or *COQ7* in the Gal-*YAH1* strain prevented the accumulation of the electroactive compound



upon depletion of Yah1p (Figure 3A and data not shown). This result suggests that the electroactive compound is part of the Q<sub>6</sub> pathway and is formed downstream of HHB.

In order to determine whether the compound is produced during the Q<sub>6</sub> biosynthetic pathway or is a degradation product of Q<sub>6</sub>, we exploited a strain which accumulates mostly DMQ<sub>6</sub> instead of Q<sub>6</sub>. This strain contains a chromosomal insertion of a sequence coding for a triple hemagglutinin (3HA) epitope tag on the 3'end of *COQ7*. The presence of the 3HA epitope on the C terminus of Coq7p likely causes the partial inactivation of the protein as evidenced by the accumulation of large amounts of DMQ<sub>6</sub> as compared to Q<sub>6</sub> (Figure 3B). Depletion of Yah1p in a Gal-*YAH1 COQ7-3HA* strain resulted in a strong decrease of DMQ<sub>6</sub> and still promoted the accumulation of the electroactive compound eluting at around 610 s (Figure 3B). This result demonstrates that this compound is not a degradation product of Q<sub>6</sub> and strongly suggests that it originates from Q<sub>6</sub> biosynthesis at a step prior to the hydroxylation of DMQ<sub>6</sub> catalyzed by Coq7p.

We purified the compound in its reduced form, analyzed it by high resolution mass spectrometry and obtained a m/z ratio of 518.43610 (M+H<sup>+</sup>). The molecular mass was submitted to different chemical formula prediction software; C<sub>36</sub>H<sub>55</sub>NO was the elemental composition which fitted best (M= C<sub>36</sub>H<sub>55</sub>NO: 517.42836; observed mass, 517.42828; ppm, 0.2) when C,H,N,O,P,S atoms were included in the calculation. In order to determine if the C<sub>36</sub>H<sub>55</sub>NO molecule contains a hexaprenyl moiety like Q<sub>6</sub>, we obtained two fragmentation spectra of the 518.4 molecular ion (M+H<sup>+</sup>) on different spectrometers. The fragments at m/z 122 and 162 are consistent with carboxyaminotropylium and carboxyaminopyrilium ions, respectively (Figure S2A). Tropylium and pyrilium ions are typically observed upon fragmentation of Q<sub>6</sub> (m/z 197 and 237) and DMQ<sub>6</sub> (m/z 167 and 207) (Padilla et al., 2004). The fragmentation observed on the spectrum in Figure S2B is consistent with the successive loss of methine, methylene or methyl groups of the hexaprenyl chain, the lightest fragment

(162.0915) corresponding to  $C_{10}H_{12}NO$ . Both fragmentation spectra are indicative of the presence of a hexaprenyl chain in the  $C_{36}H_{55}NO$  molecule.

We next characterized the purified  $C_{36}H_{55}NO$  molecule by NMR spectroscopy. The resulting  $^1H$  NMR spectrum shows well-defined peaks with some of them easily attributable to the hexaprenyl moiety (methyl, methylene and vinylic protons) (Figure 3C and experimental procedures). The aromatic region shows three massifs (6.67 to 6.61 ppm, 6.57 to 6.54 ppm and 6.52 to 6.46 ppm) of equal intensity all situated at rather high field for aromatic protons (Figure 3D). These results strongly suggest the presence of three electro-donating substituents on the aromatic ring. Moreover, the observed multiplicity associated with the value of the coupling constants unambiguously establishes both meta and para substitutions. Finally, the aromatic region of the spectrum was compared to that of the commercially available 3-methyl-4-aminophenol (Figure 3D). Both spectra were almost super-imposable, with identical pattern and coupling constants and slightly different chemical shifts. The UV-visible absorption spectra of the reduced and oxidized forms of the purified compound and of 3-methyl-4-aminophenol show identical absorbance maxima (Figure S2C and S2D). All these results unambiguously identify 3-hexaprenyl-4-aminophenol as the electroactive compound accumulated in Yah1p-deficient cells (Figure 3E). For the sake of simplicity, the numbering of the aromatic carbon atoms of the different  $Q_6$  intermediates throughout this manuscript will be as in Figure 3E.

### **pABA is the precursor of 3-hexaprenyl-4-aminophenol and competes with 4-HB for prenylation by Coq2**

The presence of a nitrogen atom in a product synthesized *in vivo* by the  $Q_6$  biosynthetic pathway was surprising. Indeed, C, H and O atoms are the only constituents of the different intermediates of Q biosynthesis described to date (Figure 1). Para-aminobenzoic

acid (pABA) is structurally close to para-hydroxybenzoic acid (4-HB), the precursor of the aromatic ring of Q (Figure 1). We therefore hypothesized that the 3-hexaprenyl-4-aminophenol synthesized in the *Yah1p*-deficient strain might originate from pABA. The accumulation of 3-hexaprenyl-4-aminophenol increased in the Gal-*YAH1* strain upon addition of pABA to YP-glucose (YPD) medium (Figure 4A) showing that pABA is the precursor of 3-hexaprenyl-4-aminophenol. We also consistently detected more 3-hexaprenyl-4-aminophenol when the Gal-*YAH1* strain was cultured in glucose synthetic complete (SC-2% glu) medium compared to YPD (compare Figure 4A and 4B). This is likely because pABA is an ingredient of synthetic medium at 0.2mg/L.

pABA being a precursor of 3-hexaprenyl-4-aminophenol suggested that pABA is prenylated by the polyprenyl transferase Coq2p, which is known to prenylate 4-HB. Decreased levels of 3-hexaprenyl-4-aminophenol were observed in the Gal-*YAH1* strain when 4-HB was added to the culture medium (Figure 4B) suggesting a competition between pABA and 4-HB for prenylation by Coq2p. Addition of 3,4-dihydroxybenzoic acid was without an effect on the accumulation of 3-hexaprenyl-4-aminophenol (data not shown).

A *coq5Δ* strain accumulates HHB, the product of the prenylation of 4-HB catalyzed by Coq2p (Figure 1)(Tran and Clarke, 2007). Esterification of HHB with diazomethane stabilizes HHB and facilitates its detection by mass spectrometry (Poon et al., 1995). 3-Hexaprenyl-4-hydroxybenzoic acid methylester (HHBme) ( $M = C_{38}H_{56}O_3$ : 560.42291; observed mass, 560.42113; ppm, 3.2) was detected spectroscopically eluting at 620 s in chromatograms of diazomethane-treated extracts of the *coq5Δ* strain (Figure 4C), and was found to be absent from extracts of the WT strain or from untreated extracts (data not shown). pABA supplementation increased the accumulation of 3-hexaprenyl-4-aminobenzoic acid methylester (HABme) ( $M = C_{38}H_{57}NO_2$ : 559.43890; observed mass, 559.43931; ppm, 0.7) eluting at 570 s in diazomethane-treated extracts of the *coq5Δ* strain (Figure 4C). HABme was

also absent from extracts of the WT strain or from untreated extracts (data not shown). It is apparent in Figure 4C that pABA supplementation increases HABme and decreases HHBme whereas 4-HB supplementation increases HHBme and decreases HABme. This shows that pABA and 4-HB are competing substrates for the prenylation reaction catalyzed by Coq2p.

### **Concomitant accumulation of 3-hexaprenyl-4-hydroxyphenol and 3-hexaprenyl-4-aminophenol by the *Yah1p*-deficient strain.**

SC medium contains pABA which efficiently competes with 4-HB for the prenylation reaction. In order to check if a compound originating from 4-HB may form within the *Yah1p*-deficient strain, we cultured the Gal-*YAH1* strain in YPD instead of SC medium (Figure 2A). Together with 3-hexaprenyl-4-aminophenol at 610 s, we detected an electroactive compound eluting at 800 s in extracts of the Gal-*YAH1* strain cultured in YPD (Figure 5A and Figure 4A). The purified compound yielded a pseudo-molecular ion at  $m/z$  519.41962 ( $M+H^+$ ) upon high resolution mass spectrometry analysis. This value is consistent with a chemical formula  $C_{36}H_{54}O_2$  ( $M= C_{36}H_{54}O_2$ : 518.41235; observed mass, 518.4118; ppm, 1.1). The fragmentation spectrum supports the presence of a hexaprenyl tail in the molecule with fragments corresponding to loss of methine, methylene or methyl groups of the hexaprenyl chain (Figure S3A). UV-Vis spectra of the  $C_{36}H_{54}O_2$  molecule are similar to the ones of methyl-1,4-benzoquinone (Figure S3B and S3C respectively). These results in addition to the fact that the  $C_{36}H_{54}O_2$  molecule is not formed in either the Gal-*YAH1 coq5Δ* or Gal-*YAH1 coq7Δ* cells (data not shown) strongly suggest that 3-hexaprenyl-4-hydroxyphenol is the electroactive compound eluting at 800 s (Figure 5B).

This conclusion was further substantiated by the finding that 4-HB is the precursor of 3-hexaprenyl-4-hydroxyphenol. Indeed, addition of 4-HB to YPD medium increased 3-hexaprenyl-4-hydroxyphenol and decreased 3-hexaprenyl-4-aminophenol in the *Yah1p*-

deficient strain (Figure 5C). In contrast, addition of pABA increased the quantity of 3-hexaprenyl-4-aminophenol as noted earlier (Figure 4A), but also decreased the production of 3-hexaprenyl-4-hydroxyphenol (Figure 5C). This competition between pABA and 4-HB for the formation of 3-hexaprenyl-4-aminophenol and 3-hexaprenyl-4-hydroxyphenol parallels the one observed for the biosynthesis of 3-hexaprenyl-4-aminobenzoic acid and 3-hexaprenyl-4-hydroxybenzoic acid (Figure 4C).

### **pABA is a precursor of Q<sub>6</sub>**

We have shown that 3-hexaprenyl-4-aminophenol and 3-hexaprenyl-4-aminobenzoic acid originate from pABA, suggesting that pABA enters the Q<sub>6</sub> biosynthetic pathway (Figure 4A and 4C). In order to check if pABA may be a precursor of Q<sub>6</sub> like 4-HB, we synthesized U-(<sup>13</sup>C)-pABA from U-(<sup>13</sup>C)-toluene in three steps (Figure S4 and Supplemental experimental procedures). As expected, culture of the Gal-*YAH1* strain in YPD containing U-(<sup>13</sup>C)-pABA predominantly yielded <sup>13</sup>C<sub>6</sub>-labeled 3-hexaprenyl-4-aminophenol at m/z 524.4 (data not shown). A WT strain was cultured in YPD supplemented with 2mg/L of U-(<sup>13</sup>C)-pABA and Q<sub>6</sub> was purified. The mass spectrum of this purified Q<sub>6</sub> showed two peaks of comparable abundance at m/z 591.4 and 597.4 (Figure 6A), corresponding respectively to unlabeled Q<sub>6</sub> and <sup>13</sup>C ring-labeled Q<sub>6</sub>. This result clearly shows that pABA is a precursor of Q<sub>6</sub> in yeast. In order for pABA to be converted into Q<sub>6</sub>, the amine group must be replaced by an hydroxyl group during the Q<sub>6</sub> biosynthetic pathway after the formation of 3-hexaprenyl-4-aminobenzoate. The *COQ7-3HA* strain mostly accumulated DMQ<sub>6</sub> instead of Q<sub>6</sub> (Figure 3B). The mass spectrum of DMQ<sub>6</sub> purified from the *COQ7-3HA* strain grown in YPD supplemented with 2mg/L of U-(<sup>13</sup>C)-pABA displayed two main ions (M+H<sup>+</sup>) m/z 561.43036 and 567.45111, which correspond to unlabeled DMQ<sub>6</sub> (M= C<sub>38</sub>H<sub>56</sub>O<sub>3</sub>: 560.42294; observed mass, 560.42254; ppm, 0.7) and to <sup>13</sup>C ring-labeled DMQ<sub>6</sub> (M= <sup>13</sup>C<sub>6</sub><sup>12</sup>C<sub>32</sub>H<sub>56</sub>O<sub>3</sub>: 566.44307;

observed mass, 566.44329; ppm, 0.4) respectively. This result demonstrates that the NH<sub>2</sub>-to-OH substitution occurs prior to the hydroxylation of DMQ<sub>6</sub> by Coq7p, the penultimate step of Q<sub>6</sub> biosynthesis.

We next cultured the W303 WT strain in synthetic medium lacking pABA. To our surprise, cells grown in pABA-free medium accumulated 8 to 10 fold less Q<sub>6</sub> than cells grown in rich (YP) medium or in synthetic medium (Figure 6B). Addition of 0.2 mg/L of pABA to pABA-free medium yields synthetic medium and restored normal levels of Q<sub>6</sub> (Figure 6B). pABA and 4-HB were equally efficient to support Q<sub>6</sub> synthesis when added to pABA-free medium. These results illustrate our conclusion that pABA is a precursor of Q<sub>6</sub> and support the idea that most of the Q<sub>6</sub> synthesized by yeast grown in synthetic medium actually originates from the pABA present in the growth medium. In order to determine whether *S. cerevisiae* uses endogenous pABA for Q<sub>6</sub> biosynthesis, we cultured the Gal-*YAH1* strain in pABA-free medium containing 2% galactose and then in pABA-free medium containing 2% glucose. 3-Hexaprenyl-4-aminophenol was detected in cell lipid extracts (data not shown), showing that endogenously synthesized pABA enters the Q biosynthetic pathway.

## Discussion

In this work, we show that the mitochondrial ferredoxin Yah1p and its reductase Arh1p, the yeast homologues of mammalian Adx and AdxR, are essential for Q biosynthesis in yeast. Strains depleted for any of these two essential proteins have a strongly reduced content of Q<sub>6</sub> and accumulate 3-hexaprenyl-4-aminophenol and 3-hexaprenyl-4-hydroxyphenol (Figure 5C). 3-hexaprenyl-4-aminophenol results from the prenylation of pABA by Coq2p whereas prenylation of 4-HB, the long known ring precursor of Q, will ultimately produce 3-hexaprenyl-4-hydroxyphenol (Figure 7 paths 1 and 4, respectively). pABA and 4-HB differ only by one substituent of benzoic acid, an amine or an hydroxyl

group, and both molecules originate in *E. coli* and yeast from chorismate, a product of the shikimate pathway. In agreement with our results, pABA has previously been shown to be prenylated by orthologs of Coq2p in *E. coli* and rat as evidenced by the observation of 3-polyprenyl-4-aminobenzoic acid (Hamilton and Cox, 1971) (Alam et al., 1975). pABA can therefore enter the Q biosynthetic pathway in many organisms but are these organisms capable of converting 3-polyprenyl-4-aminobenzoic acid into Q? Our work clearly shows that, in yeast, 3-hexaprenyl-4-aminobenzoic acid is converted to Q<sub>6</sub> since addition of U-(<sup>13</sup>C)-pABA to the growth medium of WT yeast strain promotes the biosynthesis of <sup>13</sup>C<sub>6</sub> labeled Q<sub>6</sub> (Figure 6A and Figure 7 path 2). pABA is likely not a precursor of Q in *E. coli* because a mutant strain deficient in chorismate synthetase activity synthesized Q upon supplementation of the growth medium with 4-HB but not with pABA, despite the formation of 3-polyprenyl-4-aminobenzoic acid (Hamilton and Cox, 1971). Our results with yeast warrant a thorough investigation to determine whether mammalian cells can convert pABA into Q. *In vitro* experiments by Rudney and collaborators on rat liver demonstrated that pABA is converted to 3-polyprenyl-4-aminobenzoic acid and suggested that this compound was further modified although not into Q (Alam et al., 1975). Therefore pABA has been considered an inhibitor of Q biosynthesis in mammals but only a limited decrease of Q was observed in cancer cells upon culture in a high concentration of pABA (Brea-Calvo et al., 2006).

In view of our observation that Q<sub>6</sub> levels drop dramatically when yeast is cultured in pABA-free medium, it is reasonable to postulate that most of the Q<sub>6</sub> produced by yeast cultured in SC medium originates from the pABA present in this growth medium. Our results suggest that in pABA-free medium the rate-limiting step of Q<sub>6</sub> biosynthesis is the availability of the ring precursors of Q, 4-HB and pABA. Endogenous levels of these molecules allow the synthesis of only 1.4 pmoles of Q<sub>6</sub>/mg of wet cells under our non-repressive culture conditions. One can then question the physiological significance of the high levels of Q<sub>6</sub> (12-

16 pmoles of Q<sub>6</sub>/mg of wet cells) synthesized by yeast grown in SC medium or rich medium. Actually, several authors have shown that only a small percentage of the Q<sub>6</sub> synthesized in these growth media is sufficient to sustain respiratory growth (Mollet et al., 2008; Tran et al., 2006), suggesting that, under normal conditions, Q<sub>6</sub> is in vast excess for its function as an electron carrier in the respiratory chain. Our results also provide a possible explanation as to why, contrary to *E. coli*, mutants affecting 4-HB synthesis may not abrogate yeast Q<sub>6</sub> biosynthesis and therefore not result in a respiratory deficient phenotype. Previously, authors have speculated that yeast may produce 4-HB by multiple ways; from chorismate via the chorismate pyruvate-lyase reaction similar to the *E. coli* pathway and from tyrosine like in higher eukaryotes (Clarke, 2000). However, none of the enzymes responsible for these reactions have been identified in yeast. The fact that pABA can be used in Q<sub>6</sub> biosynthesis like 4-HB and that pABA is present in standard yeast culture media imply that pABA may support biosynthesis of Q<sub>6</sub> in a yeast mutant blocked for the synthesis of 4-HB when cultured in standard conditions.

The conversion of pABA into Q<sub>6</sub> suggests an additional step in Q<sub>6</sub> biosynthesis: the replacement of the amine by an hydroxyl group at C4 (Figure 7 path 2). This reaction certainly takes place downstream of the hexaprenylation reaction catalyzed by Coq2p since 3-hexaprenyl-4-aminobenzoic acid was detected in a *coq5Δ* strain (Figure 4C). The formation of <sup>13</sup>C<sub>6</sub>-DMQ<sub>6</sub> upon supplementation of the growth medium with U-(<sup>13</sup>C)-pABA proves that the C4 aromatic NH<sub>2</sub>-to-OH conversion occurs prior to the formation of DMQ<sub>6</sub>. The accumulation of 3-hexaprenyl-4-aminophenol in the Yah1p-depleted strain suggests that this reaction occurs after the C1 hydroxylation step. Alternatively, the presence of the amine group at this stage may simply result from the lack of hydroxylation of the adjacent C5 position. Indeed, based on the example of bacterial anthranilate and aniline dioxygenases, which catalyze the conversion of aromatic amines into ortho-catechols (Beharry et al., 2003)



(Ang et al., 2007), the NH<sub>2</sub>-to-OH substitution on C4 may occur together with the hydroxylation on C5. The NH<sub>2</sub>-to-OH conversion reaction is chemically challenging and future studies will have to elucidate this newly evidenced process in Q<sub>6</sub> biosynthesis.

What is the role of Yah1p/Arh1p in Q<sub>6</sub> biosynthesis? Deficiency in any of these two proteins results in accumulation of 3-hexaprenyl-4-amino/hydroxyphenol. In order to form 3-hexaprenyl-4-amino/hydroxyphenol from 3-hexaprenyl-4-amino/hydroxybenzoic acid, the decarboxylation and hydroxylation at position 1 must occur without hydroxylation of C5 (Figure 7, paths 1 and 4). Therefore the most upstream reaction of the Q<sub>6</sub> biosynthetic pathway to be inhibited in the absence of Yah1p/Arh1p is the C5 hydroxylation of 3-hexaprenyl-4-amino/hydroxybenzoic acid. The identity of the mono-oxygenase that catalyzes the C5 hydroxylation is currently unknown. Coq6p has been initially proposed to catalyze either the C5 or C1 hydroxylations or both (Gin et al., 2003). Our results strongly argue for the involvement of Coq6p in only one of the two reactions since the C1 hydroxylation still proceeds in the Yah1p/Arh1p-depleted strains whereas the C5 hydroxylation does not. Coq6p sequence contains conserved motifs characteristic for flavin-dependent mono-oxygenases such as 4-hydroxybenzoate hydroxylase (Gin et al., 2003). Reduction of the FAD cofactor at the active site of 4-hydroxybenzoate hydroxylase has been extensively studied and shown to proceed via a direct hydride transfer from NADPH (Ballou et al., 2005). By analogy, NAD(P)H is the likely electron donor to Coq6p, making the hydroxylation catalyzed by Coq6 independent of the Yah1p/Arh1p reducing system. We thus propose that the C1 hydroxylation which is unaffected by depletion of Yah1p/Arh1p is catalyzed by Coq6p (Figure 7). This point is difficult to prove since a *coq6*Δ strain, like the other *coq3* to *coq9* null mutants, accumulates only HHB because of the instability of the Q biosynthetic complex (Gin et al., 2003; Hsieh et al., 2007). The involvement of Coq6p in the C1 hydroxylation implies that the C5 hydroxylation is catalyzed by an unknown oxygenase which may require an electron

transport machinery like Yah1p/Arh1p. This scenario would explain why the C5 hydroxylation is abrogated upon depletion of Yah1p or Arh1p.

How to explain that the reactions after the C1 hydroxylation (C2 methylation by Coq5p, C6 hydroxylation by Coq7p and O6 methylation by Coq3p) do not take place in the Yah1p-depleted strain (Figure 7 path 1 and 4)? Yah1p is likely not directly required for the methylation reactions since: (i) methylations are non redox reactions (ii) they employ S-Adenosyl-Methionine (SAM) as a methyl donor, a cofactor whose synthesis is cytosolic and does not require Fe-S containing enzymes whose maturation is generally dependent on Yah1p (Lange et al., 2000). The two O-methylation reactions can not proceed because of the lack of the two corresponding hydroxylations (C5 and C6). The absence of methylation at C2 may be explained in that the C2 carbon will be insufficiently enriched in electrons for an efficient nucleophilic attack to the methyl group of SAM in the absence of the methoxy group on C5. Alternatively, the absence of this methoxy group may prevent substrate binding and recognition by Coq5p. Finally, we envision at least two reasons that could explain the inactivity of Coq7p in the absence of Yah1p/Arh1p. On the one hand, Coq7p may not bind a substrate that lacks the C5 methoxy group and the C2 methyl group. On the other hand, Yah1p/Arh1p may provide electrons for the reduction of the di-iron center of the mono-oxygenase Coq7p, a scenario which was the founding hypothesis of this study. We could not gain any information on this point since Yah1p/Arh1p is required for the hydroxylation of 3-hexaprenyl-4-amino/hydroxybenzoic acid, a reaction upstream of the hydroxylation of DMQ<sub>6</sub> which is catalyzed by Coq7p. Further work is needed to test if Yah1p/Arh1p is the physiological reducing system of Coq7p. Answering of this question will probably require the development of an *in vitro* assay for Coq7p function, a task highly challenging given that, *in vivo*, Coq7p is part of the Q biosynthetic complex (Tran and Clarke, 2007).

In conclusion, our study has unraveled that pABA serves as a precursor of Q<sub>6</sub> similar to 4-HB whose role in Q biosynthesis was identified more than forty years ago. We also demonstrated that the yeast Adx/AdxR homologs Yah1p/Arh1p are required for the first hydroxylation reaction of the yeast Q<sub>6</sub> biosynthetic pathway, which represents a new function for these proteins. In the light of these results, it seems important to evaluate whether these new actors of yeast Q<sub>6</sub> biosynthesis play a role in mammalian Q biosynthesis.

## Significance

Coenzyme Q is a redox active lipid that functions in electron transport chains in cellular membranes and also has an important antioxidant function. Q is a prenylated benzoquinone which biosynthesis starts with the assembly of a polyprenyl chain and its conjugation to 4-HB, the precursor of the benzoquinone moiety (Turunen et al., 2004). Here we demonstrate that yeast can convert pABA into Q making of pABA a new precursor of Q, like the long-known 4-HB. Our finding thus implies that an aromatic NH<sub>2</sub>-to-OH conversion must occur to synthesize Q from pABA. The reducing system formed by the mitochondrial ferredoxin Yah1p and its associated reductase Arh1p is essential for Fe-S cluster biosynthesis (Lange et al., 2000; Li et al., 2001). This study identifies a new function for Yah1p and Arh1p in Q biosynthesis in yeast. Studies on yeast have provided the basis for the elucidation of the eukaryotic biosynthesis of Q. Sequence identity to yeast genes and complementation of yeast mutants have allowed the characterization of genes implicated in human Q biosynthesis. Mutations in five human genes have been shown to cause primary Q deficiency, which results in encephalomyopathy and severe infantile multisystemic disease. It will be of interest to define whether pABA and the orthologs of Yah1p and Arh1p are involved in mammalian Q biosynthesis.

## Experimental Procedures

**Yeast strains and culture conditions:** Yeast strains used in this study are listed in Table S1. Yeast strains were transformed using lithium acetate. A 3HA epitope tag was inserted on the 3' end of *COQ7* ORF by PCR as described previously (Longtine et al., 1998) to create the *COQ7*-3HA strain. This strain was crossed with the Gal-*YAH1* gene to isolate the *COQ7*-3HA Gal-*YAH1* strain by selecting the corresponding markers after tetrad dissection. The *coq5*Δ Gal-*YAH1* and *coq7*Δ Gal-*YAH1* strains were obtained in a similar way. In Gal-*ARH1*, the upstream region from *ARH1* was exchanged for the galactose-inducible *GAL-L* promoter by PCR mediated DNA replacement (Janke et al., 2004). Yeast media were supplemented with either 2% glucose or 2% galactose. Synthetic medium consisted in 1.7g/L YNB and 5g/L ammonium sulfate and the appropriate nutrients to complement strains auxotrophies. Synthetic complete (SC) medium was obtained by adding 2g/L Drop-out mix Synthetic minus Ade, His, Leu, Trp, Ura (US Biological) to synthetic medium. YNB without pABA and folate was from MP Biomedicals and was supplemented with 5ug/L folate. Rich YP medium was prepared as described (Sherman, 2002). Gal-*YAH1* and Gal-*ARH1* strains were maintained and precultured on galactose medium. Depletion of Yah1p was accomplished by diluting 300 fold the preculture into glucose containing medium and growing the cells for 18 hours at 30°C. Depletion of Arh1p required two subsequent dilutions in glucose medium.

**Plasmids :** *YAH1* ORF with its own promoter (380bp) and terminator (150bp) was cloned into pRS416 using XhoI and SacI. This vector served as a template to generate the C103A and C103,106S mutants by site directed mutagenesis. Sequencing was used to confirm cloning products in all created vectors. The *E. coli* strain DH5α was used for plasmid DNA amplification.

**Miscellaneous biochemical analysis:** Isolation of yeast mitochondria and immunostaining were performed as described (Diekert et al., 2001) (Harlow and Lane, 1988).

**Lipid extraction and quantification of electroactive compounds by HPLC-ECD**

**(electrochemical detection):** Lipid extraction and analyses of quinone content were essentially as described (Tran et al., 2006). 100  $\mu$ L glass beads, 50  $\mu$ L H<sub>2</sub>O and Q<sub>4</sub> (used as an internal standard) were added to mitochondria (100  $\mu$ g mitochondrial proteins) or to cell pellets (40 mg wet weight). Tubes were wrapped in foil and lipids were extracted by adding 0.6 mL methanol and 0.4 mL petroleum ether and by vortexing for 3 minutes. The phases were separated by centrifugation (3 min at 1000  $\times$  g, 4  $^{\circ}$ C). The upper petroleum ether layer was transferred to a fresh tube. 0.4 mL petroleum ether was added to the glass bead-methanol containing tube and the extraction was repeated 2 more times. The petroleum ether layers were combined and dried under nitrogen. The lipids were resuspended in 100  $\mu$ L mobile phase (98% methanol, 20 mM lithium perchlorate) and aliquots were analyzed by reversed-phase high pressure liquid chromatography with a C18 column (Betabasic-18, 5  $\mu$ m, 4.6  $\times$  150 mm, Thermo Scientific) at a flow rate of 1 mL/min. Quinones were quantified with an ESA Coulochem II electrochemical detector and a 5011A analytical cell (E1, -500 mV; E2, 500 mV). Hydroquinones present in samples were oxidized with a pre-column 5020 guard cell set in oxidizing mode (E, +550 mV). The maximum output signal of the analytical electrode E2 was set to +10 V and was recorded by a 4 channel signal recorder USB10 (Velleman instruments). The Q<sub>4</sub> external standard was used to correct for sample loss during the organic extraction based on its recovery (always greater than 85%).

**NMR analysis of 3-hexaprenyl-4-aminophenol:** NMR spectra were recorded on a Bruker EMX 300 MHz spectrometer. 3-hexaprenyl-4-aminophenol: assignable protons: <sup>1</sup>H NMR (CD<sub>3</sub>OD) 6.64 (d, <sup>3</sup>J = 8.4 Hz, H<sub>2</sub>N-C-CH, 1H), 6.55 (d, <sup>4</sup>J = 2.8 Hz, H<sub>2</sub>N-C-CH-CH-C(OH)-CH, 1H), 6.49 (dd, <sup>3</sup>J = 8.4 Hz, <sup>4</sup>J = 2.8 Hz, H<sub>2</sub>N-C-CH-CH, 1H), 5.27 (t, <sup>3</sup>J = 7.1 Hz,

Ar-CH<sub>2</sub>-CH, 1H), 5.12 (m, vinylic H, 5H), 3.21 (d,  $^3J = 7.2$  Hz, benzylic H), 2.25-1.90 (m, vinylic CH<sub>2</sub> and CH<sub>3</sub>)

**Esterification and analysis of 3-hexaprenyl-4-aminobenzoate and 3-hexaprenyl-4-hydroxybenzoate:** Dried lipid extracts from yeast cells (700mg wet weight) were resuspended in 400uL anhydrous diethyl ether. 150uL of diazomethane (0.2 M in diethyl ether) was added, the mixture was incubated for 8 minutes at room temperature and the reaction was quenched by adding 10uL of glacial acetic acid. Samples were dried under nitrogen and HPLC analysis was carried out as described for quinone content quantification except that the signal recorded was the absorbance at 273nm.

### **Mass spectrometry**

For high resolution mass spectrometry analyses, samples in methanol were diluted in 90% acetonitrile, 0.2% formic acid and were infused into the nanospray source of a discovery ORBITRAP instrument (Thermo Fischer Scientific) at a flow rate of 0.5uL min<sup>-1</sup>. The MS method used a scan range of 150-1600 m/z and was composed of MS and MS/MS events using both the Orbitrap as the analyzer (at a resolution of 30000) to get high precision on the molecular and on the fragments ions. The CID fragmentation collision energy used was set to 35 eV and the MS/MS scan range to 100-2000 m/z. QualBrowser from XCalibur was used to read the spectra.

Analyses involving coupling of HPLC to mass spectrometry were performed with an series 1100 Agilent chromatographic system associated to SCIEX API 3000 triple quadrupolar mass spectrometer. Separation was performed on an octadecylsilyl ODB Uptisphere column (150×2 mm ID, 5 µm particle size, Montluçon, France), using a gradient of acetonitrile in 2 mM ammonium formate. MS2 spectra were obtained by selecting pseudo-molecular ion in quadrupole 1 and fragmenting it in the collision cell. The fragmentation spectrum was recorded in the 50-520 mass unit range.

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## Figure legend

Figure 1: **Current model of the eukaryotic coenzyme Q biosynthetic pathway.** The names of proteins and intermediates relevant to this study are for *S. cerevisiae*. The polyprenyl diphosphate tail is assembled from one dimethylallyl pyrophosphate and 5 isopentenyl pyrophosphate in yeast or 9 in humans. Prenylation of 4-hydroxybenzoate (4-HB) by Coq2

yields 3-hexaprenyl-4-hydroxybenzoate (HHB). 7 modifications of the aromatic ring are then needed to produce coenzyme Q<sub>6</sub>. 5-demethoxyubiquinone (DMQ<sub>6</sub>) is the substrate of the mono-oxygenase Coq7p. Reactions catalyzed by unidentified or putative proteins are marked with ?.

**Figure 2: The yeast ferredoxin/ferredoxin reductase system is required for Q<sub>6</sub> biosynthesis:** A) Gal-*YAH1* cells were cultivated for 18 hours in SC medium containing either 2% galactose (gal) or 2% glucose (glu). Extracts of 10 mg (gal) and 20 mg (glu) of cells were analyzed by electrochemical detection coupled to HPLC (HPLC-ECD). Elution positions of the Q<sub>4</sub> standard, of DMQ<sub>6</sub> and Q<sub>6</sub> and of the electroactive compound (?) accumulated in Yah1p-depleted cells are indicated on the electrochromatogram. B) WT and Gal-*YAH1* cells containing an empty vector (vec) or the *YAH1* ORF were grown in SC-2% glucose for 18 hours. Extracts of 8mg of cells were analyzed by HPLC-ECD. C) WT and Gal-*ARH1* cells were cultivated for 40 h in SC-2% glucose in order to deplete Arh1p to critical levels. Mitochondria were prepared and analysed for Arh1p and Yah1p by immunostaining. Porin (Por1p) served as a loading control. D) Mitochondrial extracts (50ug of mitochondrial proteins) were analyzed by HPLC-ECD. Mitochondria were purified from WT, Gal-*YAH1* and Gal-*ARH1* cells grown in SC-2% glucose.

**Figure 3: 3-hexaprenyl-4-aminophenol is derived from Q<sub>6</sub> biosynthesis** A) Gal-*YAH1* cells and Gal-*YAH1 coq5Δ* cells were inoculated from a culture in SC-2% galactose 0.4% glucose into SC-2% glucose and grown for 18 hours. Extracts of 30 mg of cells were analyzed by HPLC-ECD. B) WT, *COQ7-3HA* and Gal-*YAH1 COQ7-3HA* cells were grown in YPD for 12 hours. Extracts of 45 mg (WT), 60 mg (*COQ7-3HA*) and 120 mg (Gal-*YAH1 COQ7-3HA*) of cells were analyzed by HPLC-ECD. C) <sup>1</sup>H NMR spectrum of the purified C<sub>36</sub>H<sub>55</sub>NO molecule

in deuterated methanol. D) Superimposition of the aromatic regions of the  $^1\text{H}$  NMR spectrum of the purified  $\text{C}_{36}\text{H}_{55}\text{NO}$  molecule displayed in panel C and of the  $^1\text{H}$  NMR spectrum of commercial 3-methyl-4-aminophenol (3M4Aphe). E) Chemical structure of 3-hexaprenyl-4-aminophenol and numbering of the aromatic carbon atoms used in the rest of the study.

Figure 4: **pABA and 4-HB compete for prenylation by Coq2p.** A) 50 mg of Gal-*YAH1* cells grown for 18 hours in YPD supplemented with pABA were extracted and analyzed by HPLC-ECD. B) 30 mg of Gal-*YAH1* cells grown for 18 hours in SC-2% glucose supplemented with 4-HB were extracted and analyzed by HPLC-ECD. C) *coq5Δ* cells were grown in 30mL YPD with the indicated concentration of pABA or 4-HB. Lipid extracts were treated with diazomethane and analyzed by HPLC. The elution profile at 273nm and the elution position and chemical structure of 3-hexaprenyl-4-aminobenzoic acid methylester (HABme) and 3-hexaprenyl-4-hydroxybenzoic acid methylester (HHBme) are shown. Areas of the peaks at 273nm for HABme and HHBme are shown in the inset (A.U., arbitrary units).

Figure 5: **Yah1p-deficient cells also accumulate 3-hexaprenyl-4-hydroxyphenol.** A) 20 mg of Gal-*YAH1* cells grown for 18 hours in YPD were extracted and analyzed by HPLC-ECD. B) Chemical structure of 3-hexaprenyl-4-hydroxyphenol. C) 30 mg of Gal-*YAH1* cells grown for 18 hours in YPD supplemented with 1 or 10mg/L 4-hydroxybenzoic acid (4-HB) or para-aminobenzoic acid (pABA) were extracted and analyzed by HPLC-ECD. 3-hexaprenyl-4-aminophenol elutes at around 610 s and 3-hexaprenyl-4-hydroxyphenol at around 800 s and areas of the peaks are shown in the inset (A.U., arbitrary units).

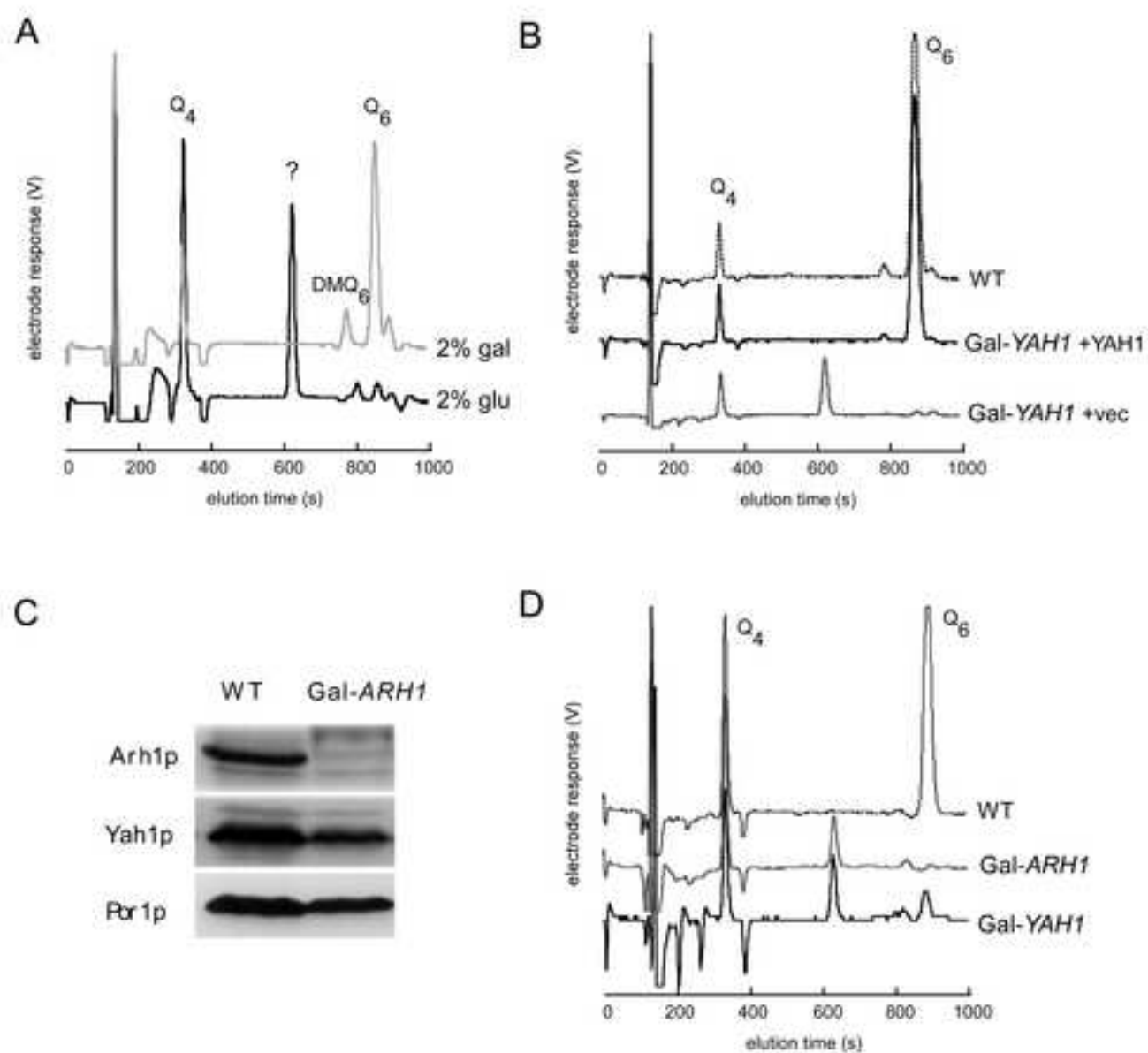
Figure 6: **pABA is a precursor of Q<sub>6</sub>.** A) Mass spectrum of Q<sub>6</sub> purified from a W303 WT strain grown in YPD medium supplemented with 2mg/L of U-( $^{13}\text{C}$ )-pABA. B) The W303 WT strain was grown in synthetic medium, synthetic medium lacking pABA or rich (YP) medium,

with 2% galactose as a carbon source. Cells were harvested in late exponential growth phase and lipids were extracted.  $Q_6$  (pmoles per mg of wet weight cell pellet) was quantified by HPLC-ECD and  $DMQ_6$  represented less than 10% of  $Q_6$  in all samples. Data are the average of at least 3 independent cultures and error bars represent the standard error of the mean.

**Figure 7: Dysfunction of the  $Q_6$  biosynthetic pathway in Yah1p/Arh1p-depleted yeast cells.** R stands for the hexaprenyl chain. Reactions blocked in the absence of Yah1p/Arh1p are shown in red. The different series of reactions are numbered path 1 to 4. Coq2p catalyzes the hexaprenylation of pABA (blue) and 4-HB to form 3-hexaprenyl-4-aminobenzoic acid and 3-hexaprenyl-4-hydroxybenzoic acid, respectively. In a WT strain, the conversion of 3-hexaprenyl-4-aminobenzoic acid into  $Q_6$  requires the  $NH_2$ -to-OH conversion on C4 which occurs prior to  $DMQ_6$  (path 2). The C5 hydroxylation of 3-hexaprenyl-4-amino/hydroxybenzoic acid catalyzed by an unidentified oxygenase is blocked in the absence of Yah1p/Arh1p (path 2 and 3). In this case, the normal  $Q_6$  biosynthetic pathway (path 2 and 3) branches off via decarboxylation of 3-hexaprenyl-4-aminobenzoic acid (path 1) and 3-hexaprenyl-4-hydroxybenzoic acid (path 4). Coq6p then synthesizes 3-hexaprenyl-4-aminophenol (path 1, framed) and 3-hexaprenyl-4-hydroxyphenol (path 4, framed). 3-hexaprenyl-4-amino/hydroxyphenol accumulate because Coq5p does not catalyze the C2 methylation and Coq7p does not catalyze the C6 hydroxylation in the absence of Yah1p/Arh1p (see discussion).

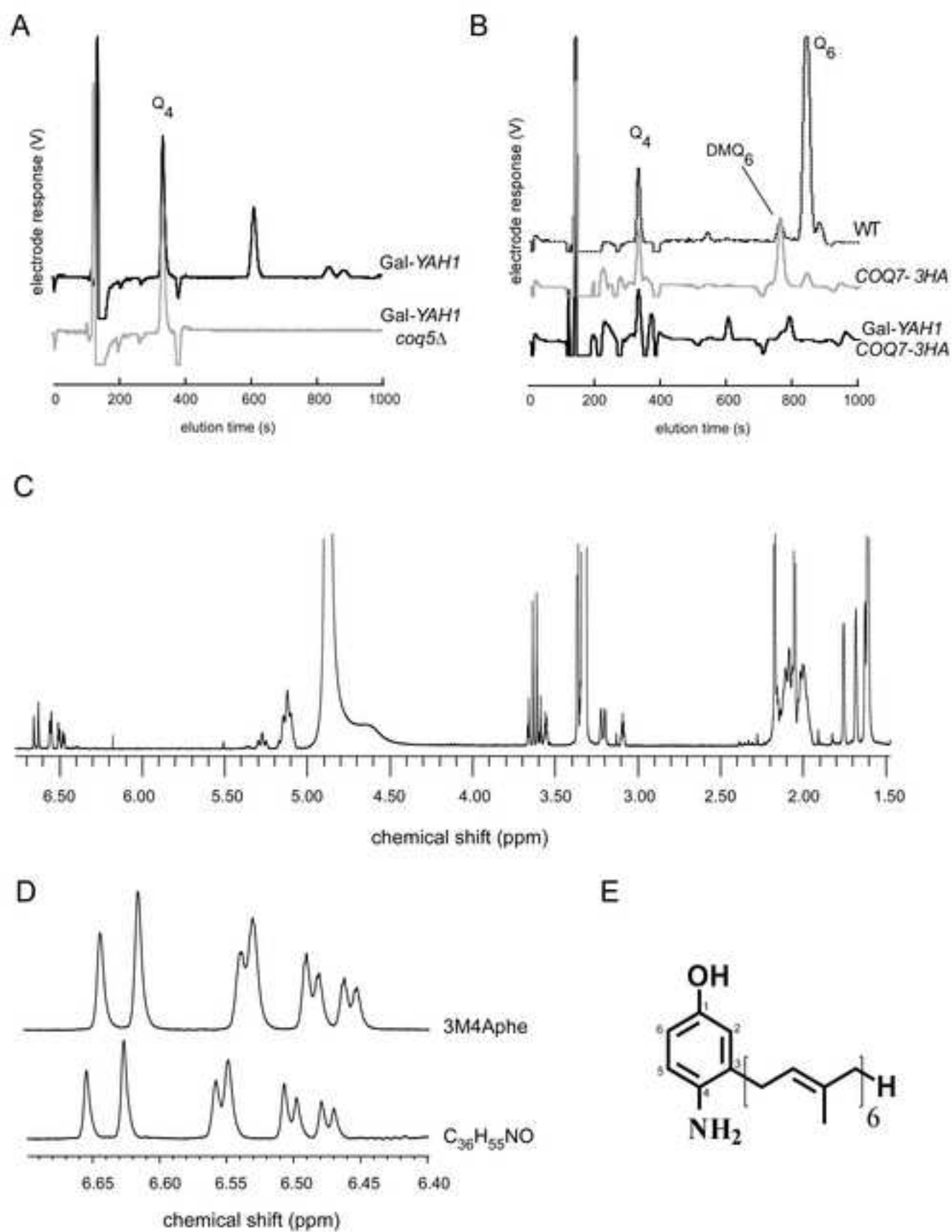


**Figure2**  
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**Figure 2**

**Figure3**  
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**Figure 3**

Figure4  
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Figure 4

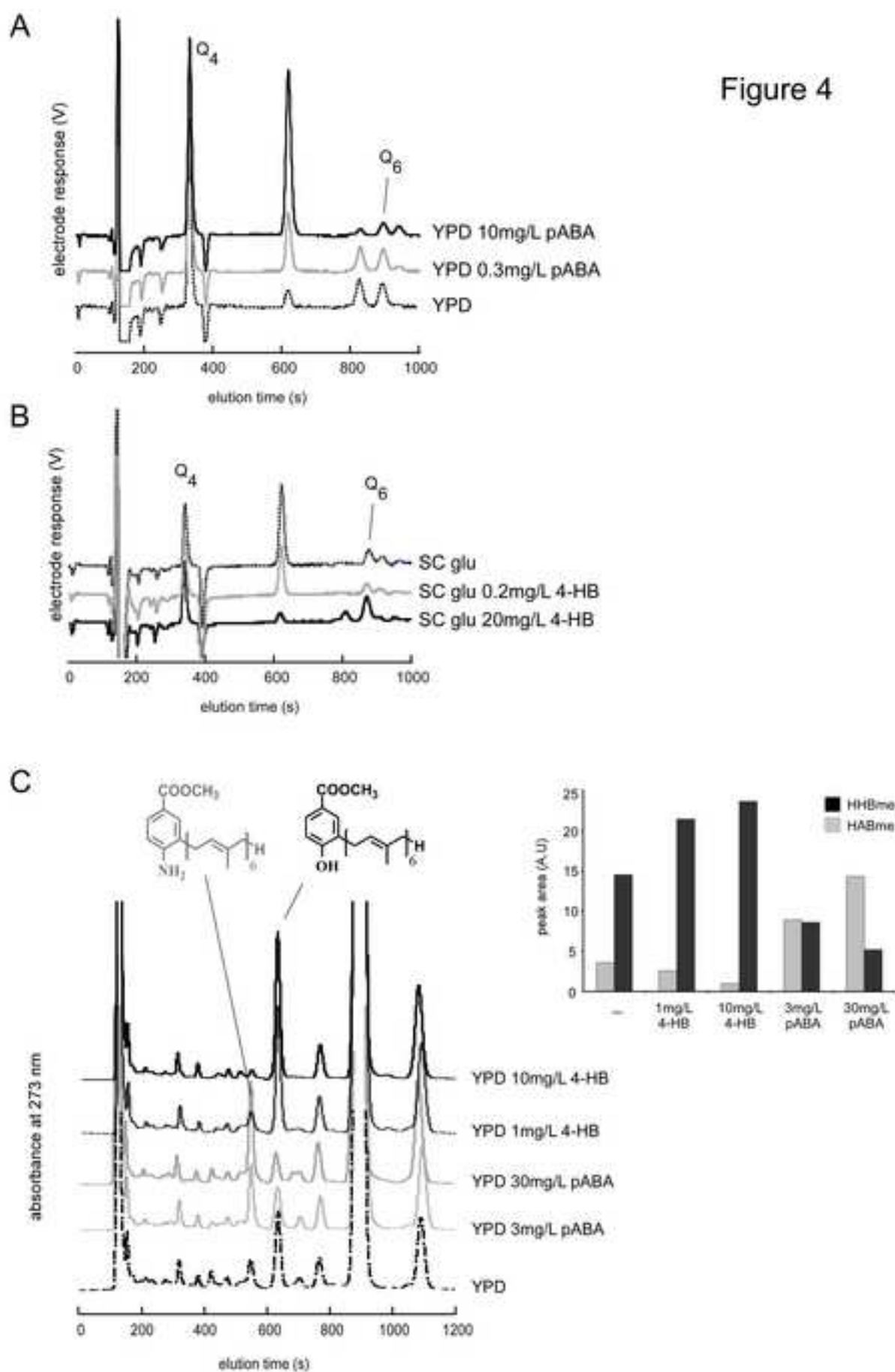




Figure5  
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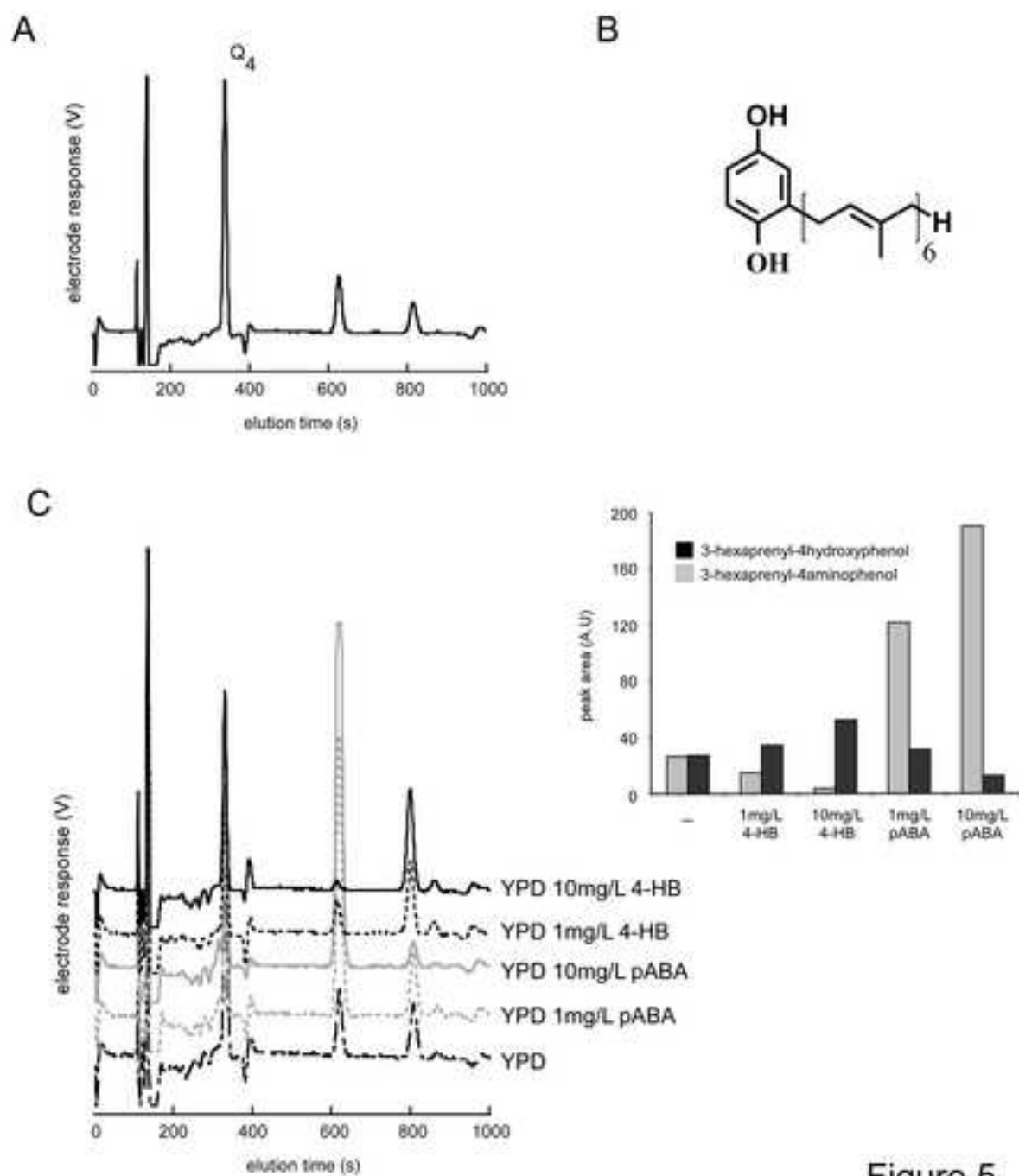


Figure 5

Figure6  
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Figure 6

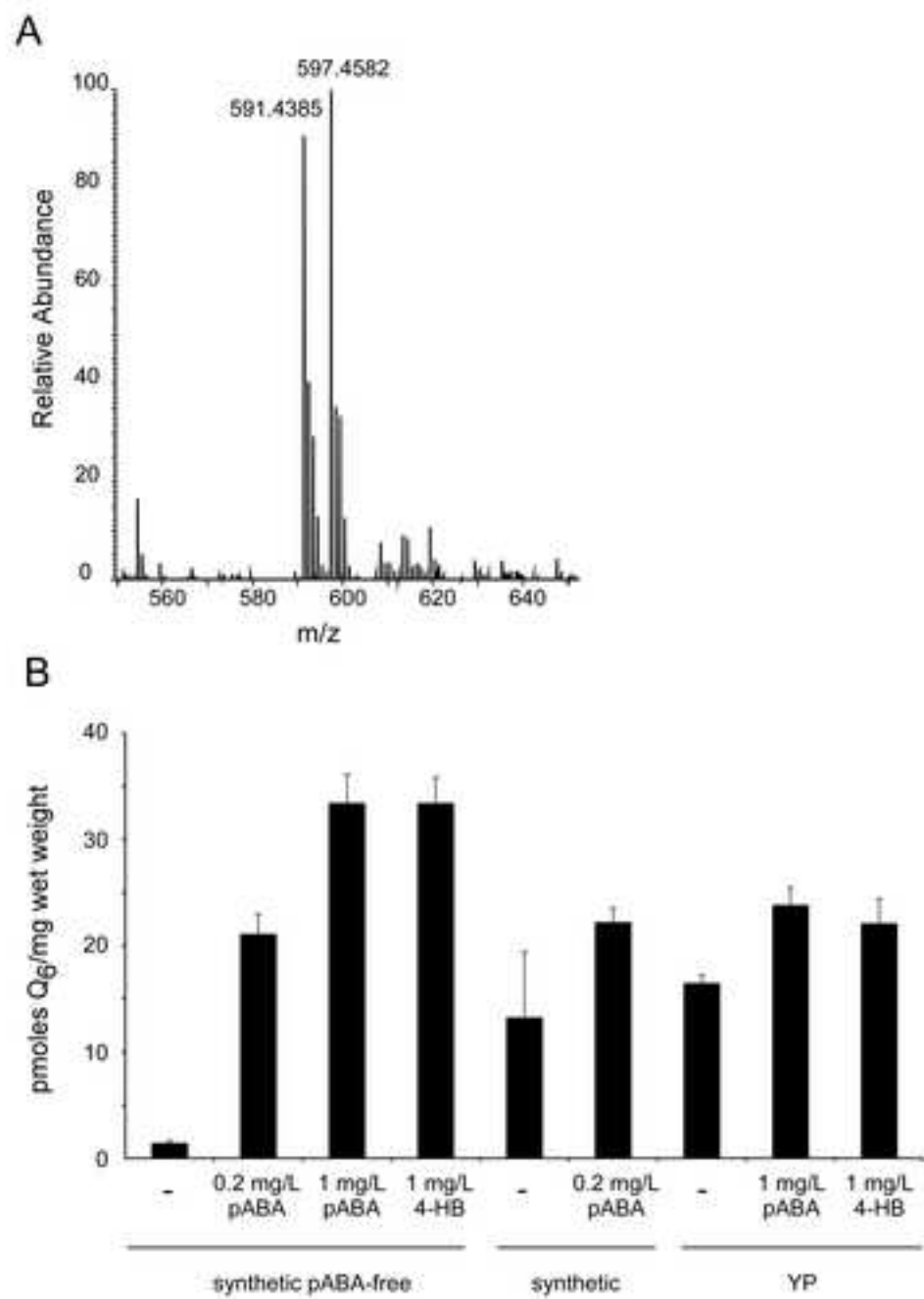
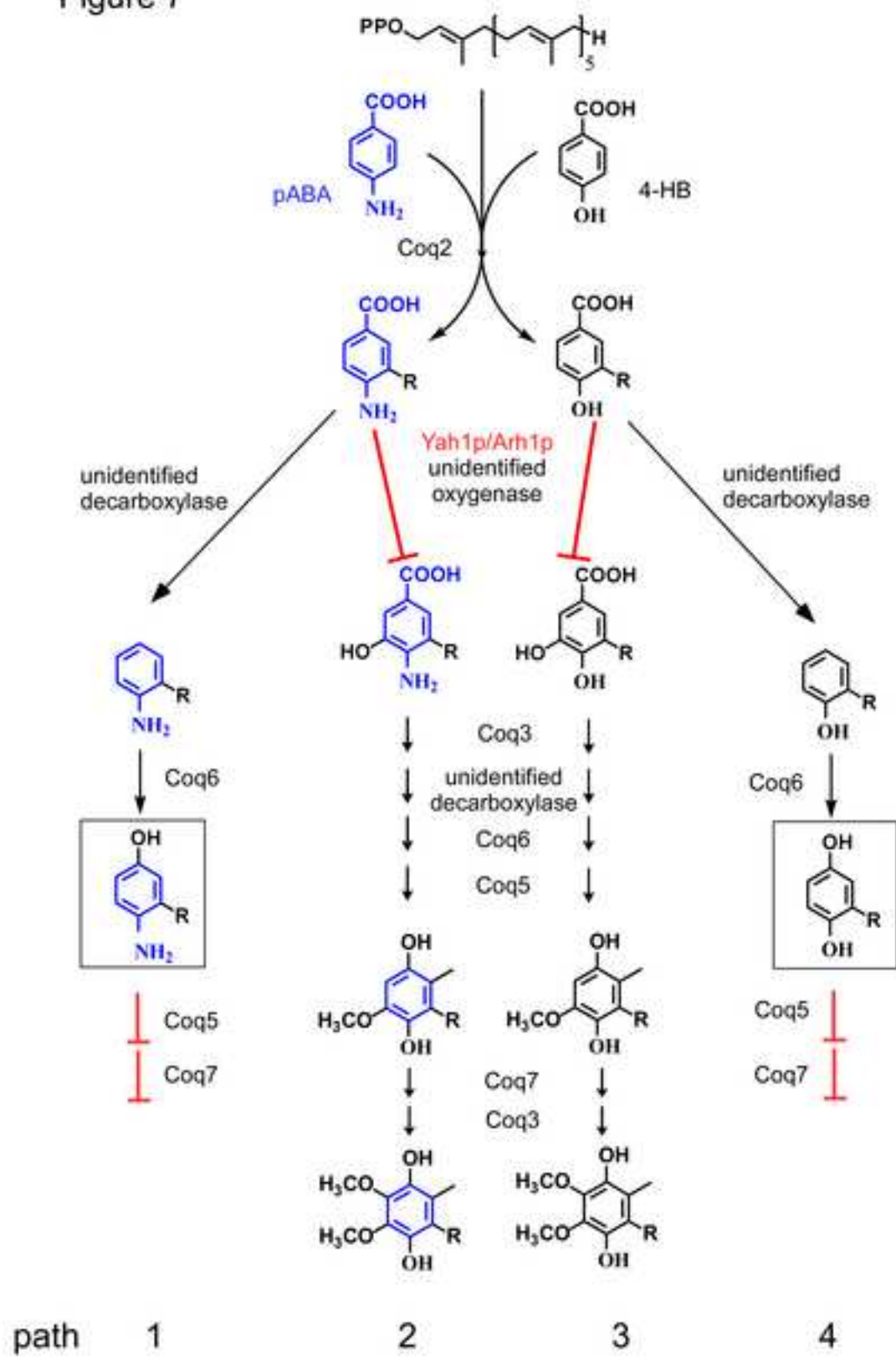


Figure 7



## **Inventory of Supplemental Information**

Supplemental information includes four figures, one table, supplemental experimental procedures and references:

Figure S1 relates to the mutants of Yah1p in Figure 2B.

Figure S2 corresponds to additional characterization of the compound displayed in Figure 3E.

Figure S3 corresponds to additional characterization of the compound displayed in Figure 5B.

Figure S4 shows the chemical synthesis of labeled pABA used in Figure 6A.

Table S1 contains the yeast strains used in this study.

Supplemental experimental procedures include the description of the chemical synthesis of labeled pABA (Figure S4) and the purification of 3-hexaprenyl-4-aminophenol (Figure 3E).

## Supplemental Figures

Figure S1

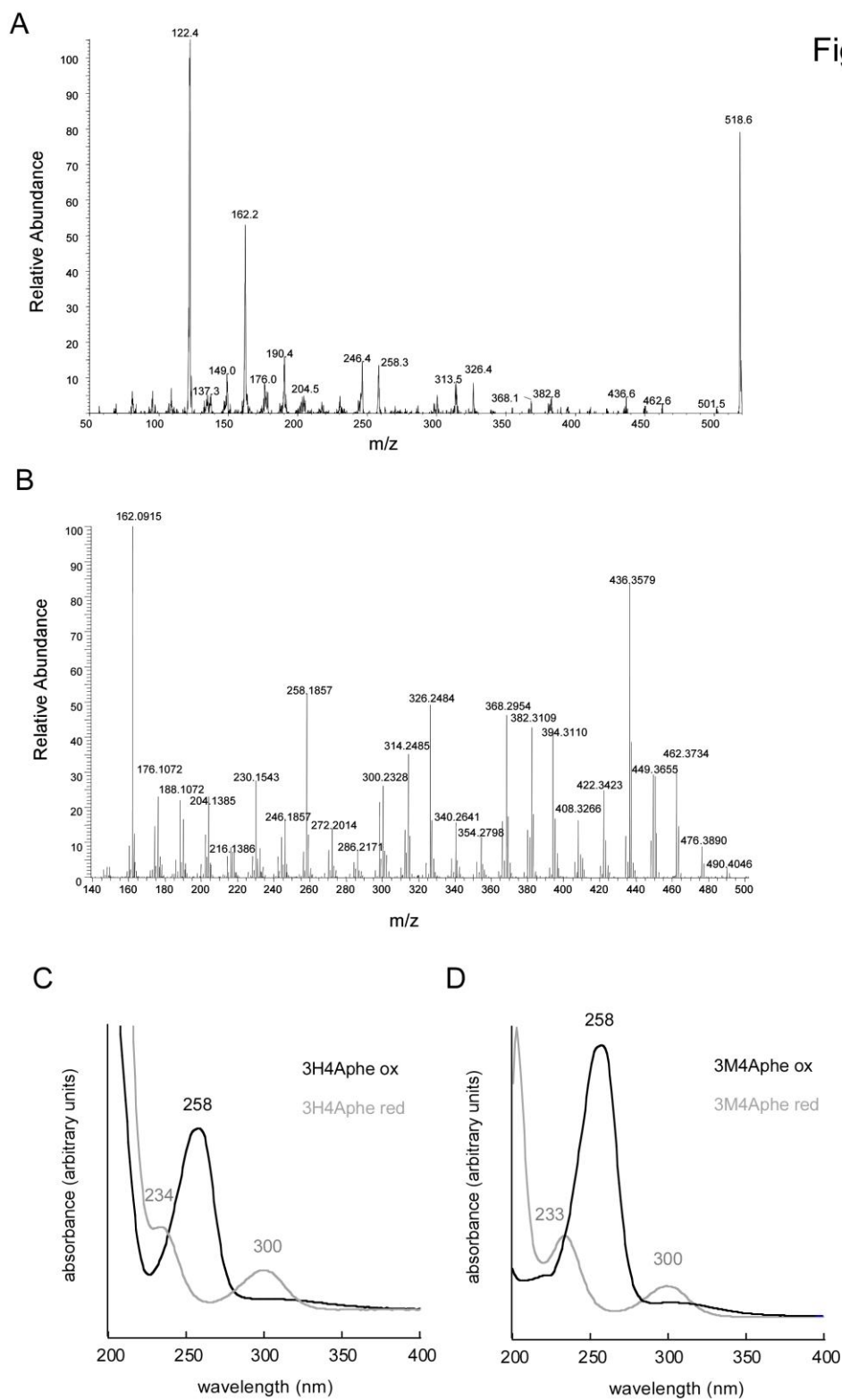
```

huFdx      -----SSS-----EDK 6
Yah1      MLKIVTRAGHTARISNIAAHLRLTSPSLLRTRTTTTTRFLPFSTSSFLNHGHLKKPKPGEE 60
                                         *: *      ::
                                         ↓      ↓
huFdx      ITVHFINRDGETLTTKGKVGDSLDDVVVENNLIDIDGFGACEGTLCSTCHILFEDHIYEK 66
Yah1      LKITFILKDGSQKTYEVCGETILDIAQGHNLDMEG--ACGGSCACSTCHVIVDPDYIDA 118
          :: ** :**. * : *::*: . :***:* ** * : *****:*. . * :

huFdx      LDAITDEENDMLDLAYGLTDRSRLGCQICLTKSMDNMTVRVPETVADARQSIDVGKTS 124
Yah1      LPEPEDDENDMLDLAYGLTETSRLGCQIKMSKDIDGIRVALPQMTRNVNN-NDFS--- 172

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Figure S2



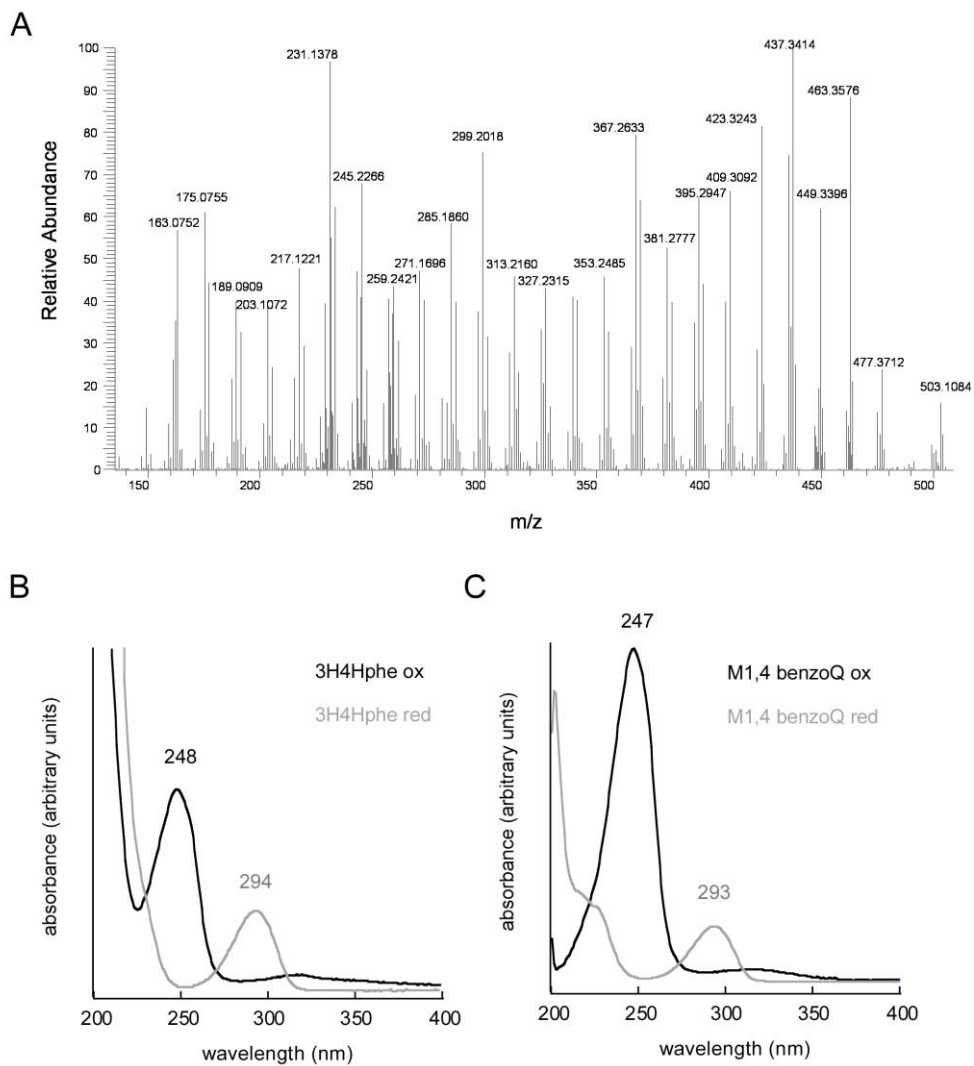
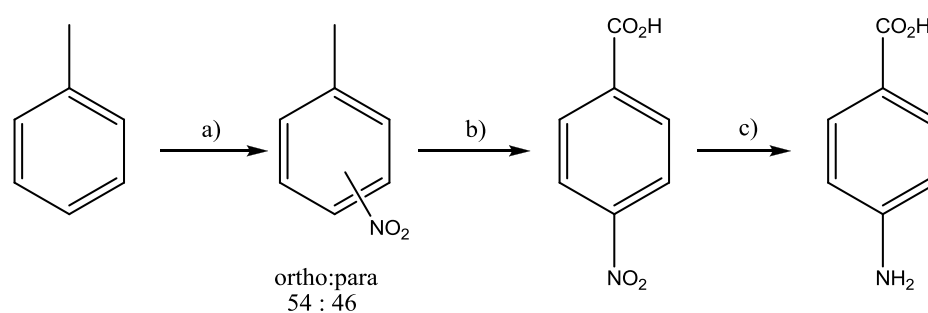


Figure S3

Figure S4



a) phosphomolybdic acid, 70% HNO<sub>3</sub>, reflux, 75 %; b) i-NaIO<sub>4</sub>, LiBr, 2% H<sub>2</sub>SO<sub>4</sub>, 95 °C, 83 %; ii- Separation;  
c) H<sub>2</sub> (1atm), Pd-C, MeOH, 99 %



Figure S1: **Sequence alignment of *S. cerevisiae* mitochondrial ferredoxin Yah1 and the human ferredoxin (huFdx).** The alignment was generated using Clustalw. The conserved cysteines 52 and 55 shown to be important to coordinate the 2Fe-2S cluster in huFdx are pointed (Xia et al., 1996). The corresponding cysteines 103 and 106 (in red) have been mutated in Yah1.

Figure S2: **Fragmentation spectra and UV-Vis spectra of the purified C<sub>36</sub>H<sub>55</sub>NO molecule.** A) Fragmentation spectrum obtained on a SCIEX API 3000 mass spectrometer. The ions at m/z 122 and 162 correspond respectively to carboxyaminotropylium and carboxyaminopyriliium ions. B) Fragmentation spectrum obtained on a discovery ORBITRAP spectrometer. The fragments whose high resolution mass are indicated correspond to loss of methine, methylene or methyl groups of the hexaprenyl chain. C) A similar quantity of purified 3-hexaprenyl-4-aminophenol (3H4Aphe) was injected on the HPLC column with the precolumn electrode set at +550mV (ox) or -500mV (red). The UV-Vis spectra of the oxidized (ox) and reduced (red) forms of 3H4Aphe were recorded at the apex of the elution peak of the product. The absorption maxima of the spectra are indicated. D) UV-Vis spectra of 1 nmole of 3-methyl-4-aminophenol (3M4Aphe) after chromatography on the HPLC column with the precolumn electrode set at +550mV (ox) or -500mV (red).

Figure S3: **Fragmentation spectrum and UV-Vis spectra of the purified C<sub>36</sub>H<sub>54</sub>O<sub>2</sub> molecule.** A) Fragmentation spectrum obtained on a discovery ORBITRAP spectrometer. The fragments whose high resolution mass are indicated correspond to loss of methine, methylene or methyl groups of the hexaprenyl chain. B) UV-Vis spectra of a similar quantity of purified

3-hexaprenyl-4-hydroxyphenol (3H4Hphe) after chromatography on the HPLC column with the precolumn electrode set at +600mV (ox) or -500mV (red). C) UV-Vis spectra of 1 nmole of methyl-1,4-benzoquinone (M1,4benzoQ) after chromatography on the HPLC column with the precolumn electrode set at +600mV (ox) or -1000mV (red).

Figure S4: **Chemical synthesis of U-(<sup>13</sup>C)-p-aminobenzoic acid.** The detailed protocol is described in the supplemental experimental procedures.

**Table S1:** *S. cerevisiae* strains used in this study

Strain	Genotype	Source
W303	MATa <i>ade2-1 his3-1,15 leu2-3,112 trp1-1, ura3-1</i>	Invitrogen
Gal-YAH1	W303 genotype, Gal-YAH1:: <i>LEU2</i>	(Lange et al., 2000)
W303 <i>coq5</i> Δ	W303 genotype <i>coq5</i> Δ:: <i>HIS3</i>	(Barkovich et al., 1997)
DY5113 (W303)	MATa <i>ade2-1 his3-1,15 leu2-3,112 trp1</i> Δ, <i>ura3-1</i>	(Keller et al., 2005)
COQ7-3HA	DY5113 genotype COQ7-3HA:: <i>TRP1</i>	This work
COQ7-3HA gal-YAH1	DY5113 genotype COQ7-3HA:: <i>TRP1</i> , Gal-YAH1:: <i>LEU2</i>	This work
<i>coq5</i> Δ gal-YAH1	W303 genotype <i>coq5</i> Δ:: <i>HIS3</i> , Gal-YAH1:: <i>LEU2</i>	This work
<i>coq7</i> Δ gal-YAH1	<i>his3, ura3, coq7</i> Δ::KanMX4, Gal-YAH1:: <i>LEU2</i>	This work
Gal-ARH1	W303 genotype Gal-ARH1:: <i>natNT2</i>	This work

## Supplemental experimental procedures

**Synthesis of U-( $^{13}\text{C}$ )-p-aminobenzoic acid:** To a solution of 300 mg of U-( $^{13}\text{C}$ )-toluene (3.03 mmol) in chloroform (25 mL) were successively added 599  $\mu\text{L}$  of 70%  $\text{HNO}_3$  (2.2 equiv.) and 1.014 g (2.2 equiv.) of phosphomolybdic acid. The resulting solution was refluxed for 5 days then washed with water and dried over  $\text{Na}_2\text{SO}_4$ . Concentration under vacuum afforded 327 mg (2.27 mmol, 75 %) of nitrotoluene as a mixture of two regioisomers (ortho: para ratio, 54:46) unseparable by silica gel chromatography and pure enough to be used in the next step without further purification.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ) 8.50-8.30 (m, 1H), 7.90-7.50 (m, 4H), 7.30-6.75 (m, 3H), 2.60 (dq,  $^2J_{13\text{C-H}} = 129 \text{ Hz}$ ,  $^3J_{13\text{C-H}} = 9.3 \text{ Hz}$ ,  $^4J_{13\text{C-H}} = 5.1 \text{ Hz}$ ,  $^4J_{13\text{C-H}} = 5.1 \text{ Hz}$ , 3H, ortho isomer), 2.47 (dq,  $^2J_{13\text{C-H}} = 127 \text{ Hz}$ ,  $^3J_{13\text{C-H}} = 10.2 \text{ Hz}$ ,  $^4J_{13\text{C-H}} = 5.4 \text{ Hz}$ ,  $^4J_{13\text{C-H}} = 5.4 \text{ Hz}$ , 3H, para isomer).

To a solution of 207 mg (1.5 mmol) of U-( $^{13}\text{C}$ )-nitrotoluene (as a 54:46 mixture of ortho and para isomers) in 7 mL of 2% aqueous  $\text{H}_2\text{SO}_4$  were successively added 642 mg (2 equiv.) of  $\text{NaIO}_4$  and 287 mg (2 equiv.) of  $\text{LiBr}$ . After 30 hours at  $95^\circ\text{C}$ , 2 additional equiv of both  $\text{NaIO}_4$  and  $\text{LiBr}$  were added and the resulting solution was stirred for additional 20 hours at  $95^\circ\text{C}$ . The aqueous solution was then extracted with  $\text{EtOAc}$  (3 times) and the resulting organic was successively washed with saturated solution of  $\text{Na}_2\text{S}_2\text{O}_3$ , twice with water, brine, dried over  $\text{Na}_2\text{SO}_4$  then concentrated under vacuum to afford 209 mg (1.24 mmol, 83%) of the mixture of ortho and para U-( $^{13}\text{C}$ )-nitrobenzoic acid as a pale yellow solid. Recrystallisation by slow evaporation of diethylether from a solution of the crude product in diethylether resulted in the formation of a mixture of two kinds of crystals. Manual separation

allowed the isolation of 17 mg of pure U-( $^{13}\text{C}$ )-*para*- nitrobenzoic acid.  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ ) 8.7-8.5 (m, 2H), 8.12-7.95 (m, 2H).

A suspension of 17 mg (97.7  $\mu\text{mol}$ ) of U-( $^{13}\text{C}$ )-*p*-nitrobenzoic acid and 5 mg of 10 % Pd-C in 2 mL of methanol was purged with hydrogen then vigorously stirred under  $\text{H}_2$  atmosphere (1 bar) for two days. After filtration and evaporation of the solvent under vacuum, 14 mg (97.2  $\mu\text{mol}$ , 99 %) of U-( $^{13}\text{C}$ )-*p*-aminobenzoic acid were obtained as a pale yellow solid and was pure enough to be used without further purification.  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ ) 7.75 (dm,  $^2J_{^{13}\text{C}-\text{H}} = \text{Hz}$ , 2H), 6.72 (dm,  $^2J_{^{13}\text{C}-\text{H}} = \text{Hz}$ , 2H),  $^1\text{H}$ - $^{13}\text{C}$  decoupling NMR spectrum: 7.63 (d,  $J_{\text{AB}} = 8.1 \text{ Hz}$ , 2H), 6.50 (d,  $J_{\text{AB}} = 8.7 \text{ Hz}$ , 2H)

**Purification of 3-hexaprenyl-4-aminophenol:** A total of 130g of Gal-YAH1 cells (wet weight) from a culture in SC medium 2% glucose with 10mg/L pABA were used for lipid extraction as described above with volumes scaled up accordingly and without addition of  $\text{Q}_4$ . The petroleum ether extract (400mL) was dried under vacuum. The extract was resuspended in 10mL methanol and lithium perchlorate was added to a final concentration of 40mM. The precipitate was eliminated by centrifugation and the product was purified by multiple injections on the C18 column using 98% methanol 20mM lithium perchlorate at 0.5mL/min with the precolumn electrode set first at -450mV, then at 700mV and finally at -500mV. The retention time of the product changed from 10min in the reduced form to 16 min in the oxidized form. Finally, the product was injected in its reduced form on the C18 column using 100% methanol as a mobile phase and collected by monitoring the absorbance at 300nm. The pure product was dried under vacuum and resuspended in deuterated methanol for subsequent NMR analysis.

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